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Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli*

Key words *Alcaligenes eutrophus* * *Escherichia coli* * *Clostridium kluyveri* * biodegradable polyesters * polyhydroxyalkanoic acids * poly(4HB) * 4-hydroxybutyric acid metabolism * PHA * PHA synthase * 4-hydroxybutyric acid:CoA transferase

Abbreviations *PHA* polyhydroxyalkanoic acid * *3HB* 3-hydroxybutyric acid * *4HB* 4-hydroxybutyric acid * *CDW* cellular dry weight * *IPTG* isopropylthiogalactosid

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Abstract The aim of this study was the production of the homopolyester poly(4-hydroxybutyric acid), poly(4HB), with recombinant strains of *Escherichia coli*. Wild type strains and other strains of *E. coli* widely used are not able to produce poly(hydroxyalkanoic acids), PHA, and can not utilize 4-hydroxybutyric acid as a carbon source. Accordingly, hybrid plasmids of pBluescript vectors were constructed which harbored the *Alcaligenes eutrophus* PHA synthase gene (*phaC*) and the *Clostridium kluyveri* orfZ putatively encoding a 4-hydroxybutyric acid-coenzyme A transferase. A 3.5-kbp genomic *SmaI/ApaI*-fragment from *A. eutrophus*, which comprises *phaC*, and a 1.8-kbp genomic *ApaI/EcoRI* fragment from *C. kluyveri*, which contained orfZ, were inserted into the *SmaI*- and *EcoRI*-sites of the vectors pKS^- and pSK^-, respectively. The two resulting plasmids pSKSE5.3 and pKSSE5.3 comprising *phaC* and orfZ colinear or antilinear to *lacZ*, respectively, were transformed into *E. coli* XL1-Blue. Recombinant strains synthesized the homopolyester poly(4HB), when the cells were cultivated in Luria Bertani broth and if glucose plus 4-hydroxybutyric acid were provided as carbon sources. If glucose was omitted, a copolyester of 3-hydroxybutyric acid and 4-hydroxybutyric acid was accumulated. The homopolyester poly(4HB) was also accumulated during cultivation of these strains in M9 mineral salts medium containing glucose plus 4-hydroxybutyric acid as carbon sources. Poly(4HB) could amount up to approximately 80 % (w/w) of the cell dry matter if *E. coli* XL1-Blue harboring pKSSE5.3 was cultivated in M9-mineral salts medium and if the cultures were not sufficiently supplied with oxygen. A copolyester of 3HB and 4HB was accumulated by the same strain if γ-butyrolactone was used instead of 4-hydroxybutyric acid. If levulinic acid, 4-hydroxyvaleric acid or γ-valerolactone were used as carbon sources, only very low amounts of PHA were accumulated which did not contain 4-hydroxyalkanoic acids as constituents.

Introduction

A large variety of bacteria is able to synthesize polyesters of hydroxyalkanoic acids and to accumulate these polyhydroxyalkanoic acids (PHA) as insoluble cytoplasmic inclusions. After the discovery of a polyester consisting of 3-hydroxybutyric acid (3HB) in cells of *Bacillus megaterium* (Lemoigne 1926) poly(3HB) remained for a long period the only known PHA. Since the seventies an steadily increasing number of new constituents have been identified in these bacterial storage polyesters, and at present more then 100 different hydroxyalkanoic acids are known to occur as building blocks of PHA. Most of these constituents were compiled in a recent review (Steinbüchel and Valentin 1995). These polyesters have attracted a considerable interest of academia and industry since they are biodegradable thermoplastics and/or elastomers that are considered for various technical applications in industry and agriculture as well as for medical applications (Hocking and Marchessault 1994, Müller and Seebach 1993).

The PHA synthase structural gene from *Alcaligenes eutrophus* (*phaC*$_{Ae}$) has been cloned and characterized at the molecular level in several laboratories (for a review see Steinbüchel and Schlegel 1991). It was demonstrated that *phaC*$_{Ae}$ in combination with other genes conferred the ability to synthesize poly(3HB) not only to many bacteria, which do not synthesize this polyester such as e. g. *Escherichia coli* (Steinbüchel and Schlegel 1991) but also to *Saccharomyces cerevisiae* (Leaf et al. 1996), to plants such as *Arabidopsis thaliana* (Poirier et al. 1992) and *Gossypium hirsutum* (John & Keller 1996) and even to cells from the insect *Spodoptera frugiperda* (Williams et al. 1996).

This study aimed at the establishment of a pathway to produce poly(4HB) homopolyester in recombinant cells of *E. coli*. This bacterium is unable to synthesize poly(3HB) or any other PHA and to accumulate such polyesters as cytoplasmic inclusions. Furthermore, it can not use 4HB as a carbon source (Söhling and Gottschalk 1996), and no pathways are known in *E. coli* which could generate 4HB or 4HB-CoA. However, a poly(3HB) synthesis pathway was repeatedly established in several laboratories in recombinant strains of *E. coli* which expressed the *A. eutrophus* PHA operon comprising the structural genes of β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase or related clusters of genes from other bacteria. It has been shown that *A. eutrophus* is able to synthesize PHA containing 4HB as constituent and multiple evidence has been obtained that the PHA synthase from this bacterium, which is encoded by *phaC*$_{Ae}$, can use 4HB-CoA as a substrate (Kunioka et al.

1988). In addition, evidence was obtained that a genomic fragment cloned from *Clostridium kluyveri*, which comprised a cluster of genes the translational products of which are required for the conversion of succinate to 4HB-CoA during fermentation of ethanol plus succinic acid to butyric acid (Söhling and Gottschalk 1996, Kenealy and Waselefsky 1985). This gene cluster contained an open reading frame, which was referred to as orfZ and putatively encodes a 4HB-CoA transferase (Söhling 1993). Therefore, we investigated in this study whether a combination of $phaC_{Ae}$ and orfZ could confer poly(4HB) biosynthesis to *E. coli* .

Materials and methods

Bacterial strains and plasmids used in this study

Escherichia coli XL1-Blue (Bullock et al. 1987) and the plasmid pBluescriptKS and SK (Stratagene, La Jolla, Cal. USA), pSK2665 (Schubert et al. 1991) and pCK3pSK (Söhling and Gottschalk 1996) were used in this study.

Media and cultivation conditions

Cells of *A. eutrophus* were cultivated at 30°C either in complex nutrient broth medium or in a mineral salt medium according to Schlegel et al. (1961), whereas cells of *E. coli* were cultivated at 37°C in complex Luria-Bertani broth or in M9-minerals salts medium (Sambrock et al. 1989). Additions of antibiotics, which were prepared according to Sambrock et al. (1989), and of carbon sources, which were sterilized by filtration, are described in the text. Cultivations were done either on solidified media, which were obtained by the addition of 1.5 % (wt/vol) agar, or in liquid media in Erlenmeyer flasks that were incubated on a rotary shaker.

Isolation and analysis of polyesters

For quantitative determination of PHA and for the analysis of the constituents of PHA, 3 - 5 mg lyophilized cell material or the isolated polyester were subjected to methanolysis in the presence of 15 % (vol/vol) sulphuric acid, and the resulting hydroxyacyl methylesters were analyz d by gas chromatography as described in detail by Brandl et al. (1988) and Timm et al. (1990).

PHA were isolated from lyophilized cells by extraction with chloroform in a Soxhlet apparatus. The polyester was precipitated from the chloroform solution by the addition of 10 vol of ethanol, and the precipitate was subsequently separated from the solvents by filtration. Remaining solvents were removed by exposure of the polyester to a stream of air. For further purification, the polyester was again dissolved in chloroform and precipitation in ethanol was repeated.

Nucleic acid techniques

Plasmid DNA and DNA restriction fragments were isolated and analysed by standard methods compiled in a laboratory manual (Sambrock et al. 1989). Restriction enzymes, ligases and other enzymes manipulating DNA molecules were used according to the instructions provided by the manufacturers of these enzymes.

***** Physical characterization of poly(4HB) samples

analysis of molecular weight and polydispersity by GPC; analysis of melting points, rate of crystallization, δH_m, E_a by DSC experiments. *****

***** These paragraphs will not included in this publication, it was only included to compile data for the patent application. They might be included in a joint publication of Münster and Monsanto in which the properties of the recombinant poly(4HB) are described. *****

Results

Construction of plasmids

A 3.5-kbp *SmaI/ApaI* restriction fragment comprising the entire PHA synthase structural gene (*phaC*$_{Ae}$) plus 878 of 1,221 bp of the 5´region of the ß-ketothiolase structural gene (*phaA´*$_{Ae}$) from *A. eutrophus*, and which was referred to as SA35, was isolated from the hybrid plasmid pSK2665 that was cloned previously (Schubert et al. 1991). In addition, a 1.8-kbp *ApaI/Eco*RI restriction fragment comprising the entire orfZ$_{Ck}$ from *C. kluyveri*, and which was referred to as AE18, was isolated from the hybrid plasmid pCK3pSK that was cloned

recently (Söhling and Gottschalk 1996). Both fragments were ligated to the pBluescript vectors KS⁻ and SK⁻, which had been treated with *Sma*I plus *Eco*RI, and the ligation products (pKSSE5.3 and pSKSE5.3, respectively) were transformed to *E. coli* XL1-Blue. Fig. 1 shows the construction of pKSSE5.3, which contained $phaC_{Ae}$ and $orfZ_{Ck}$ adjacent but antilinear to the *lacZ* promoter. In pSKSE5.3 (not shown) $phaC_{Ae}$ and $orfZ_{Ck}$ were located downstream plus colinear to the *lacZ* promoter. All constructs were controlled by agarose gel electrophoresis for the presence of the expected restriction fragments.

For control experiments, fragment SA35 alone was ligated to pBluescript KS⁻ or to pBluescript SK⁻ resulting in the plasmids pKSSA35 ($phaC_{Ae}$ plus $phaA'_{Ae}$ adjacent but antilinear to the *lacZ* promoter, Fig. 1) or pSKSA35 ($phaC_{Ae}$ plus $phaA'_{Ae}$ downstream and colinear to the *lacZ* promoter, not shown in Fig. 1), respectively. For the same reason, fragment AE18 alone was ligated to pBluescript KS⁻ or to pBluescript SK⁻ resulting in the plasmids pKSAE18 ($orfZ_{Ck}$ downstream and colinear to the *lacZ* promoter, not shown in Fig. 1) and pSKAE18 ($orfZ_{Ck}$ adjacent but antilinear to the *lacZ* promoter, Fig. 1), respectively.

Synthesis of poly(4HB) from 4-hydroxybutyric acid in recombinant strains of *E. coli*

The recombinant strains of *E. coli* XL1-Blue were cultivated at 37°C as batch cultures in Erlenmeyer flasks either using the LB complex medium supplemented with 4-hydroxybutyric acid alone or in combination with glucose as additional carbon sources or the M9 mineral salts medium containing 4-hydroxybutyric acid plus glucose as carbon sources. The hybrid plasmids pKSSE5.3 and pSKSE5.3, which comprised $phaC_{Ae}$, $phaA'_{Ae}$ plus $orfZ_{Ck}$, conferred to *E. coli* the capability to synthesize and accumulate a homopolyester of 4HB if the strains were cultivated in the presence of 4HB plus glucose as carbon source (Table 1). Light microscopic examination of the cells revealed cytoplasmic inclusions, and the harvested cells were more whitish than strains not harboring pKSSE5.3 or pSKSE5.3. If glucose was omitted as a cosubstrate the amount of PHA in the cells was approximately in the same range but a copolyester consisting of 3-hydroxybutyric acid and 4HB, poly(3HB-*co*-4HB), rather than poly(4HB) homopolyester occurred in the cells. At the very beginning obviously only 4HB was incorporated into the PHA; however, after approximately one day of cultivation the cells began to incorporate increasing amounts of 3HB, and after four days the molar fraction of 3HB had increased to approximately 70% (Table 2). If glucose was provided as the sole carbon source not any PHA was accumulated. Therefore, the addition of 4-hydroxybutyric acid to the medium as a carbon source was essential to obtain poly(4HB), and glucose provided as a cosubstrate prevented the incorporation of 3HB into PHA.

Gas chromatographic analysis of the derivatives obtained from washed and lyophilized whole cells gave two major compounds in the chromatogrammes exhibiting retention times of 9.3 and 20.1 and one minor compound exhibiting a retention time of 24.1 min. According to former analysis (H. E. Valentin and A. Steinbüchel unpublished data), these compounds represented most probably γ-butyrolactone, the methylester of 4HB and the methyl ether of 4HB, respectively, that were also obtained if only 4-hydroxybutyric acid were subjected to acidic methanolysis.

The amount of poly(4HB) accumulated by the cells depended very much on the plasmid present in the recombinant *E. coli* XL1-Blue, the medium and the cultivation conditions. Recombinant strains harboring plasmid pKSSE5.3 accumulated always significantly more poly(4HB) as compared to those harboring pSKSE5.3 in LB complex medium as well as in M9 mineral salts medium (Table 1). The addition of IPTG to cultures of *E. coli* XL1-Blue harboring pSKSE5.3 did not significantly influence the amount of polyester. With both plasmids the amount of poly(4HB) accumulated by the cells was always higher in M9-mineral salts medium than in LB complex medium. Supplementation of oxygen to the cultures seems to be crucial for the amount of poly(4HB) accumulated by the cells. This became obvious from experiments in which the ratio of the medium volume to the volume of the Erlenmeyer flask was varried. Using 250 ml-Erlenmeyer flask the amount of poly(4HB) increased tremendously if the volume of the medium was increased from 50 to 100 ml independantly whether the recombinant strains harbored pSKSE5.3 or pKSSE5.3 (Table 1). If the volume of the medium was further increased to 150 ml the amount of accumulated poly(4HB) decreased.

All plasmids, which contained only $phaC_{Ae}$ plus $phaA'_{Ae}$ (pKSSA35 and pSKSA35) or only $orfZ_{Ck}$ (pKSAE18 and pSKAE18), did not confer to *E. coli* the capability to synthesize detectable poly(4HB) or of any other PHA if 4HB or glucose alone or in combination were used a carbon sources and independent from the volume of the medium or whether IPTG was added or not.

None of the recombinant strains of *E. coli* obtained in this study was able to grow in liquid or on solidified M9 minerals salts medium containing 4-hydroxybutyric acid as sole carbon source.

Properties of poly(4HB) from recombinant *E. coli*

In order to confirm accumulation of poly(4HB) by the recombinant strains and to isolate the polyester from the cells, *E. coli* XL1-Blue (pKSSE5.3) was cultivated in M9 mineral salts medium at a larger scale. 2 l-Erlenmeyer flasks containing 800 ml medium supplemented with 0.4 % (wt/vol) sodium 4-hydroxybutyrate plus 1 % or 2 % glucose were inocculated with a preculture of 50 ml cells and incubated on a rotary shaker for 72 h. This gave approximately 1.9 or 2.5, respectively, of dried cells and gas chromatographic analysis of the whole cells indicated a poly(4HB) content of approximately 80 % (wt/wt). From these cells 0.8 or 1.1 g PHA could be extracted with chloroform and precipitated with ethanol, respectively. Therefore, only 52 or 55 % of the polyester could be recovered from the cells. This discrepancy could be explained by incomplete extractions of the cells (see also below) since the extracted cells still contained some poly(4HB) and by losses during the precipitation of the polymer. The isolated material gave in the gas chromatogramme only those signals typic for 4HB. Thus it was confirmed that the cells had accumulated poly(4HB) homopolyester.

As it was observed recently for poly(4HB) produced by a PHA-leaky mutant of *A. eutrophus* JMP222 and recombinant strains of this mutant harboring extra copies of the *A. eutrophus* PHA synthase operon (Steinbüchel et al. 1994), also the poly(4HB) produced by the recombinant strain of *E. coli* employed in this study was extracted from lyophilized cells at a much lower rate than for example poly(3HB). Poly(4HB) from the recombinant strain precipitated in the presence of an excess of ethanol from the chloroform solution as a highly fibrous material which easily and almost quantitatively ended up on a glass rod if the latter was used for stirring during precipitation. As the poly(4HB) from the strains of *A. eutrophus* JMP222 (Steinbüchel et al. 1994), poly(4HB) isolated from recombinant *E. coli* cells was a white and elastic material.

*****Gel permeation chromatography experiments done with two samples of poly(4HB) homopolyester isolated from two independent batches of cells of recombinant strains of *E. coli* revealed molecular weights (M_w) of $1.75 \cdot 10^6$ and $1.85 \cdot 10^6$, respectively, with relative low polydispersities (M_w/M_n) of 1.45 and 1.48, respectively. Therefore, the molecular weights of these poly(4HB) samples were significantly higher than the molecular weights of poly(4HB) isolated from strains of *A. eutrophus* JMP222; in addition the polydispersity of these samples was much lower (Steinbüchel et al. 1994). The two poly(4HB) samples exhibited melting points (T_m) of 67.6 and 63.1 °C. The values for δH_m and E_a for the two samples were 45.7 and 44.3 J/g or 69.8 and 83.8 KJ/mol, respectively. At 70°C both polyester samples exhibited a slow rate for crystallization (> 30 min). *****

***** These paragraphs will not included in this publication, it was only included to compile data for the patent application. They might be included in a joint publication of Münster and Monsanto in which the properties of the recombinant poly(4HB) are described. *****

Synthesis of PHA from γ-butyrolactone, levulinic acid, 4-hydroxyvaleric acid or γ-valerolactone in recombinant strains of *E. coli*

Carbon sources related to 4-hydroxybutyric acid were also investigated with respect to the formation of PHA by the recombinant strains. For this purpose, cells were cultivated in two different stages. In the first stage the cells were grown for 64 h in the complete M9 minerals salts medium containing 0.5 % (wt/vol) glucose plus 0.1 % (wt/vol) sodium 4-hydroxybutyrate as carbon sources. These cells were then washed with fresh M9 mineral salts medium and transferred to 250 ml-Erlenmeyer flasks containing 100 ml ammonium-free M9 mineral salts medium. By this the density of the cell suspension was diluted approximately 1:1 as compared to the density in the preculture. In this second stage the cells were cultivated for 72 h on a rotary shaker. If *E. coli* XL1-Blue (pKSSE5.3) was cultivated in M9 mineral salts medium containing 0.5 % (wt/vol) glucose plus 0.4 % (wt/vol) γ-butyrolactone as carbon sources, only low amounts of PHA (usually below 10% of CDW, wt/wt) were accumulated (Table 3). However, these cells did not accumulate poly(4HB) homopolyester but synthesized a copolyester consisting of 3HB and 4HB, with 4HB as a minor constituent (usually below 30 %, mol/mol).

If in these experiments γ-butyrolactone was replaced by 0.4 % (wt/vol) sodium levulinate or by 0.2 % (wt/vol) sodium 4-hydroxyvalerate or by 0.2 % (wt/vol) γ-valerolctone, only very low amounts of PHA never exceeding 4 % of the CDW were synthesized and accumulated. Gas chromatographic analysis revealed 3HB as the only constituent; 4HB, 3-hydroxyvaleric acid (3HV) or 4-hydroxyvaleric acid (4HV) could be never detected (Table 3).

Cells of *E. coli* XL1-Blue (pKSSE5.3) were also cultivated in single stage experiments as batch cultures in 250 ml Erlenmeyer flasks containing 100 ml complete M9 minerals salts medium plus 0.5 % /wt/vol) glucose plus 0.1 to 0.4 % (wt/vol) γ-butyrolactone as carbon sources. This time the cells accumulated significantly more PHA, and the polyester consisted of 4HB as the only detectable constituent (Table 3). With 0.4 % (wt/vol) sodium 4-hydroxybutyrate in the medium for example poly(4HB) contributed 16.1 % (wt/wt) to the CDW. If sodium levulinate instead of sodium 4-hydroxybutyrate was used as a second carbon

source in addition to 0.5 % (wt/vol) glucose no PHA was detected in the cells.

Discussion

This is the first report on the synthesis and accumulation of PHA containing 4HB as constituent by a recombinant strain of *E. coli*. The formation of this polyester depended strictly dependant on the presence of the PHA synthase structural gene from *A. eutrophus* and orfZ from *C. kluyveri*.. After the demonstration of poly(3HB) homopolyester (Schubert et al. 1988, Slater et al. 1988) and poly(3HB-*co*-3HV) copolyester (Slater et al. 1992) formation by recombinant strains of *E. coli* poly(4HB) can be now also produced in *E. coli*..

The experiments demonstrated that $phaC_{Ae}$ is functionally expressed from the plasmid constructs from its own promoter and independent from the *lacZ* promoter as it has been shown repeatedly in the past (e. g. Steinbüchel and Schlegel 1991, Schubert et al. 1991). The experiments also provided evidence that $orfZ_{Ck}$ was functionally expressed in the recombinant strains. Whether expression of $orfZ_{Ck}$ occurred from its own promoter or whether a polycistronic mRNA comprising the transcripts of $phaC_{Ae}$, $phaA'_{Ae}$ and $orfZ_{Ck}$ was formed remains to be elucidated and requires further analysis in particular the identification of the $orfZ_{Ck}$ promoter. The heterologous expression of these genes established in *E. coli* a two-step pathway converting the substrate and carbon source 4-hydroxybutyric acid into poly(4HB) homopolyester. From the results of these experiments it can be clearly concluded that $orfZ_{Ck}$ must encode an enzyme which is able to convert 4HB to 4HB-CoA. This is achieved either by an ATP-dependent thiokinase type of reaction utilizing free coenzyme A or by a coenzyme A transferase type of reaction which utilizes acetyl-CoA as donor. The latter is more likely to occur since this type of reaction occurs frequently in anaerobic bacteria.

Although 4-hydroxybutyric acid could definitely not be utilized as a sole carbon source for growth, it was obviously converted into an intermediate which could give rise to the formation of 3HB-CoA and subsequently to the incorporation of 3HB besides 4HB into the accumulated PHA as revealed by experiments in which 4-hydroxybutyric acid was provided as the only additional carbon source during cultivation in Luria Bertani broth. The steps of this pathway in *E. coli* are not known and have to be investigated. This pathway is obviously suppressed in the presence of glucose since under these conditions poly(4HB) homopolyester was accumulated. This make sense for the cells since the putative intemediates will be much more readily available from glucose than from 4-hydroxybutyric acid.

The absence of a foreign functionally active β-ketothiolase and an NADPH-dependent acetoacetyl-CoA reductase in the recombinant strains of *E. coli* used in this study allowed the production of poly(4HB) homopolyester from 4-hydroxybutyric acid even if glucose was present since no D(-)-3-hydroxybutyryl-CoA is formed from acetyl-CoA (Maloy and Nunn 1981). Furthermore, since *E. coli* is unable to use 4-hydroxybutyric acid as a carbon source they can not convert 4HB into central intermediates of the metabolism. This is only possible for *E. coli* if the complete *C. kluyveri* pathway for the conversion of succinate to 4-hydroxybutyrate is expressed and is obviously also functioning in the opposite direction (Söhling and Gottschalk 1996).

Since it was shown in this study that 4HB containing PHA are in recombinant strains of E. coli also obtained from γ-butyrolactone, *E. coli* must express an enzyme which hydrolyses the lactone to 4-hydroxybutyric acid. γ-Butyrolactone is much cheaper than 4-hydroxybutyric acid and provides therefore an advantages for the production of 4HB containing PHA. The absence of 4-hydroxyalkanoic acid containing PHA in recombinant cells of *E. coli* that were cultivated on levulinic acid, 4-hydroxyvaleric acid or γ-valerolactone may indicate that the orfZ$_{Ck}$ translational product is specific for 4-hydroxybutyric acid or that no transport of these carbon sources into the cells or no cleavage of γ-valerolactone can occur.

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Table 1. Accumulation of poly(4HB) by recombinant strains of *E. coli* XL1-Blue from 4-hydroxybutyric acid

Medium	Carbon source	volume of medium (ml)	PHA content (% of CDW)	accumulated polyester
<u>with pSKSE5.3</u>				
LB + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	50	3.3	poly(4HB)
LB + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	100	10.5	poly(4HB)
LB + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	150	8.8	poly(4HB)
M9 + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	100	16.9	poly(4HB)
<u>with pKSSE5.3</u>				
LB	0.5 % glucose 0.4 % 4HB (sodium salt)	50	4.3	poly(4HB)
LB	0.5 % glucose 0.4 % 4HB (sodium salt)	100	40.8	poly(4HB)
LB	0.5 % glucose 0.4 % 4HB (sodium salt)	150	20.4	poly(4HB)
M9	0.5 % glucose 0.4 % 4HB (sodium salt)	100	58.5	poly(4HB)
<u>with pSKSA35</u>				
LB + ITPG	0.5 % glucose 0.4 % 4HB (sodium salt)	50 - 150	nd	-
M9 + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	100	nd	-
<u>with pKSAE18</u>				
LB + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	50 - 150	nd	-
M9 + IPTG	0.5 % glucose 0.4 % 4HB (sodium salt)	100	nd	-

Cells were cultivated at 37°C for 72 h in one-stage cultivation experiments in 250 ml Erlenmeyer flasks as described in the text that were incubated on a rotary shaker (220 Upm). The cultures were inocculated with 0.04 vol of an over night preculture in Lurua Bertani broth containing 0.5 % (wt/vol) glucose plus 0.4 % (wt/vol) 4-hydroxybutyric acid. At the end of the experiment the cells were harvested, washed with tap water, lyophilized and analysed for PHA content and composition by gas chromatography.

Abbreviations and symbols: IPTG, isopropylthiogalactosid; nd, not detectable; -, not relevant;

Table 2. Accumulation of poly(3HB-*co*-4HB) by recombinant strains of *E. coli* XL1-Blue from 4-hydroxybutyric acid

Strain and Carbon source(s)	Incubation time (h)	PHA content (% of CDW)	PHA composition (mol%) 3HB	4HB
E. coli XL1-Blue (pSKSE5.3)				
in LB medium with 0.5 % Glucose plus 0.4 % Na-4-hydroxybutyrate	20	13.3	nd	100
	44	13.1	11	89
	72	11.1	55	45
	94	15.1	64	36
in LB medium with 0.4 % Na-4-hydroxybutyrate	20	20.2	nd	100
	44	20.6	72	28
	72	10.2	61	39
	94	21.9	66	34
E. coli XL1-Blue (pKSSE5.3)				
in LB medium with 0.5 % Glucose plus 0.4 % Na-4-hydroxybutyrate	20	14.6	nd	100
	44	13.6	nd	100
	72	7.0	nd	100
	94	15.9	7	93
in LB medium with 0.4 % Na-4-hydroxybutyrate	20	22.9	nd	100
	44	21.4	62	38
	72	23.7	63	37
	94	29.8	64	36

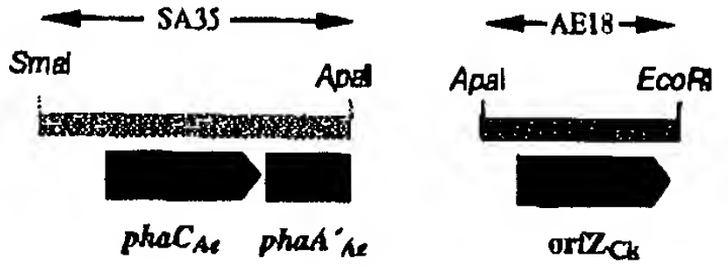
Cells were cultivated at 37°C in 50 ml Luria Bertani complex medium, which contained the indicated carbon source, in 300 ml Erlenmeyer flasks. At the indicated times samples were withdrawn and subjected to gas chromatographic analysis of the polyester content and composition. Abbreviations and symbols: nd, not detectable; PHA, polyhydroxyalkanoic acids; 3HB, 3-hydroxybutyric acid; 4HB, 4-hydroxybutyric acid; LB Luria Bertani; CDW, cellular dry weight

Table 3. Cultivation of *E. coli* XL1-Blue (pKSSE5.3) on other precursor substrates for the incorporation of 4-hydroxyalkanoic acids in PHA

		PHA content	PHA composition (mol%)			
Medium	Carbon source	(% of CDW)	3HB	3HV	4HB	4HV
<u>two-stage cultivation experiments</u>						
M9*	0.5 % glucose 0.4 % γ-butyrolactone	9.6	71	nd	29	nd
M9*	0.5 % glucose 0.4 % Na-levulinate	4.2	100	nd	nd	nd
M9*	0.5 % glucose 0.4 % Na-4-hydroxyvalerate	1.5	100	nd	nd	nd
M9*	0.5 % glucose 0.4 % γ-valerolactone	2.7	100	nd	nd	nd
<u>one-stage cultivation experiments</u>						
M9	0.5 % glucose 0.1 % γ-butyrolactone	8.0	nd	nd	100	nd
M9	0.5 % glucose 0.2 % γ-butyrolactone	13.9	nd	nd	100	nd
M9	0.5 % glucose 0.4 % γ-butyrolactone	16.1	nd	nd	100	nd
M9	0.5 % glucose 0.2 % Na-levulinate	nd	nd	nd	nd	nd
M9	0.5 % glucose 0.4 % Na-levulinate	nd	nd	nd	nd	nd

Cells were cultivated at 37°C for 72 h in one- or two-stage cultivation experiments as described in the text. At the end of the experiment the cells were harvested, lyophilized and analysed for PHA content and composition by gas chromatography.

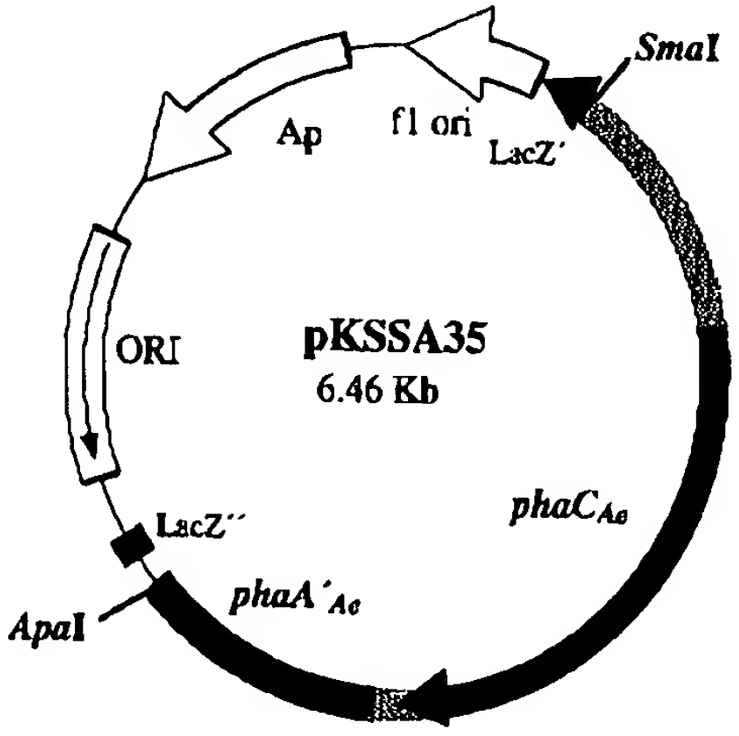
Abbreviations and symbols: M9, regular mineral salts medium; M9*, ammonium-free M9 mineral salts medium; nd, not detectable

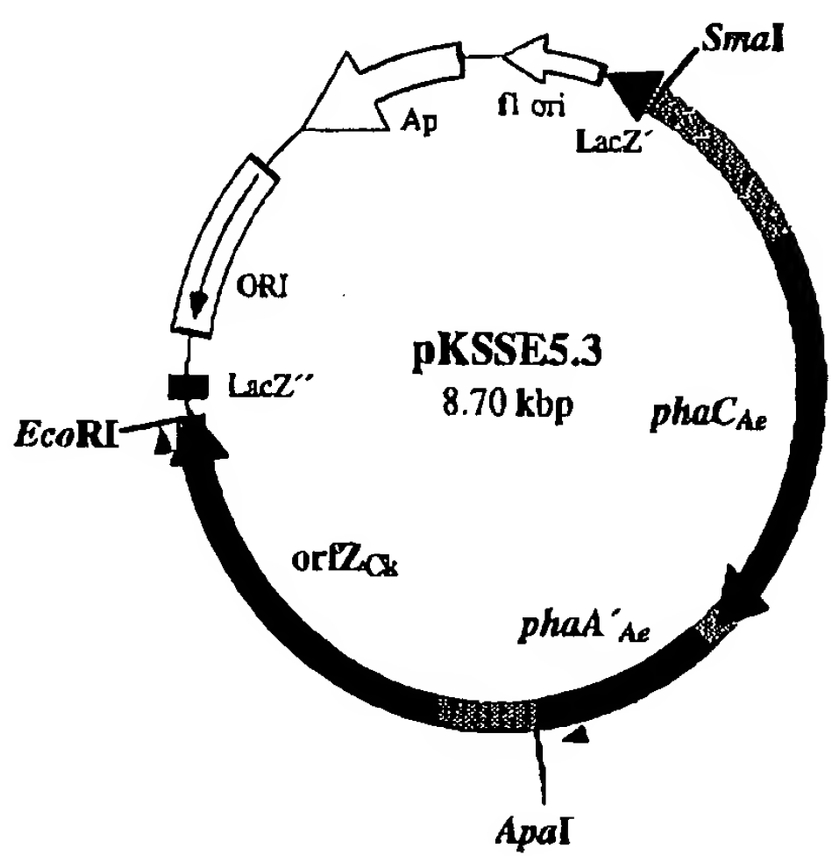


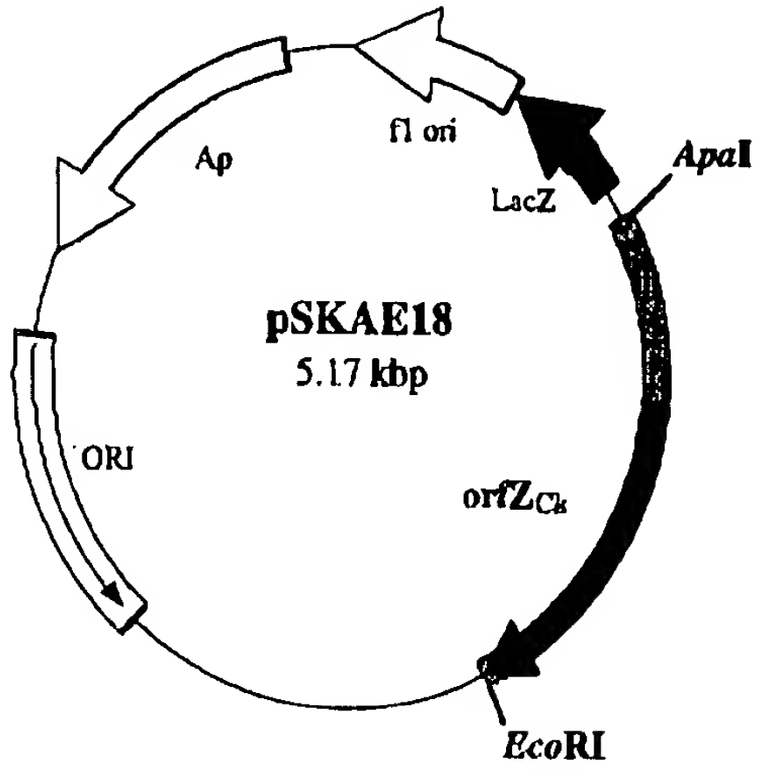
Ligation of the isolated genomic *A. eutrophus* fragment SA35 with *Sma*I- and *Apa*I-digested pBluescript KS⁻ -DNA

Ligation of the isolated genomic *fragments A. eutrophus* SA35 and *C. kluyveri* AE18 with *Sma*I- and *Eco*RI-digested pBluescript KS⁻ -DNA

Ligation of the isolated genomic *C. kluyveri* fragment AE18 with *Sma*I- and *Apa*I-digested pBluescript SK⁻ -DNA







List of putative claims

1. A new pathway for the formation of poly(4HB) in recombinant *Escherichia coli*

(i) Combination of the structural genes *phaC* from *Alcaligenes eutrophus* plus orfZ from *Clostridium kluyveri* as it has been already shown in this study to establish a two-step pathway from 4HB to poly(4HB).

(ii) Instead of *phaC* from *A. eutrophus* in principle any other PHA-MCL synthase structural gene, which can be functionally expressed in *E. coli* can be used. This includes also those PHA synthases which consists of two different subunits (PhaC + PhaE) like the PHA synthases from anoxygenic Chromatiaceae (e. g. *Thiocapsa pfennigii, Chromatium vinosum, Thiocystis violacea*)

(iii) Instead of orfZ from *C. kluyveri*, genes for any other fatty acid:CoA transferase which utilizes e. g. acetyl-coenzyme A as coenzyme A donor and which is functionally expressed in *E. coli*, can be used.

(iv) Instead of orfZ from *C. kluyveri*, genes for any 4-hydroxybutyrate thiokinase, which utilizes free conenzyme A plus ATP and which is functionally expressed in *E. coli* can be used.

2. A new pathway for the formation of poly(4HB) in other recombinant bacteria

(1-i) to (1-iv) can be formulated accordingly if E. coli is replaced by the name of a different bacterium. In principle, this can be almost any bacterium for which gene transfer systems are available

3. Variation of the precursor substrate for poly(4HB)

(i) use of γ-butyrolactone instead of 4-hydroxybutyric acid

(ii) use of 1,4-butanediol instead of 4-hydroxybutyric acid (If the diol is catabolized by oxidation via the corresponding aldehyde to 4-hydroxybutyric acid as it has been shown in e. g. strains of *A. eutrophus*)

(iii) all variations of the game listed under 1. and 2. can be considered

4. Variation of the precursor substrate for other 4-hydroxyalkanoic acid containing PHA

(i) instead of 4-hydroxybutyric acid, γ-butyrolactone or 1,4-butanediol also 4-hydroxyvaleric acid, γ-valerolactone or 1,4-pentandiol may be used (will depend on physiological background, specificity of PhaC and OrfZ or homologs)

(ii) an extension could be also made for longer-chain length 4-hydroxyalkanoic acids and the corrsponding lactones and diols.

(iii) all variations of the game listed under 1. and 2. can be considered

5. Inclusion of genes for a lactone hydr lyzing enzymes

(i) If a functionally expressed gene for a lactone hydrolyzing enzyme is included into the systems described under 1. and 2. cleavage of the lactone to the corresponding hydroxyalkanoic acid is guaranteed and will occur at a much faster rate than upon spontaneous hydrolysis or upon hydrolysis by side activities of other non-specific enzymes.

As a consequence, a three-step biosynthesis pathway starting from the lactone is directly established.

(ii) consider effect on all other claims listed under 1., 2. 3. or 4.

6. Copolyesters containing 4HB as only one of two or more constituents

(i) By feeding a cosubstrate in addition to 4-hydroxybutyric acid as a second carbon source, other hydroxyalkanoic acids in addition to 4HB may be incorporated. Which further constituents are incorporated will depend on the physiological background, on the chemical structure of the cosubstrate, on the cultivation conditions. By variation of the concentration of the cosubstrate the molar fractions of the additional comonomers can be varried. Almost anything is possible !

(ii) By controlled expression of foreign genes for ß-ketothiolase and acetoacetyl-CoA reductase, the availibility of e. g. 3-hydroxybutyryl-CoA for the PHA synthase can be controlled. This could for example result in the controlled incorporation of 3HB as comonomer into a copolyester in the recombinant strains outlined under 1.

7. Genetic approaches for the production of poly(4HB) homopolyesters or of copolyesters containing 4HB as one constituent from unrelated carbon sources

The strategies described under 1. to 6. will only result in the incorporation of 4HB or other 4-hydroxyalkanoic acids into storage polyesters if 4-hydroxybutyric acid or γ-butyrolactone or 1,4-butanediol or corrsponding homologous compounds are provided as carbon source.

In order to obtain these polyesters also from any other carbon sources and preferentially those which are available as renewable resources the following strategies, which add further elements to the *phaC* plus orfZ constructs, will be applied:

(i) 2-oxoglutarate >1> Succinate semialdehyde >2> 4HB >3> 4HB-CoA >4> poly(4HB)

#	Enzyme	Source
>1>	2-oxoglutarate decarboxylase	*Leuconostoc oenos* *Euglena gracilis*
>2>	4HB-dehydrogenase	*C. Kluyveri* (*4hbD*) *A. eutrophus* (*gbd*)
>3>	4HB-CoA transferase	*C. kluyveri* (orfZ)
>4>	PHA synthase	*A. eutrophus* (*phaC*)

(ii) Succinate >1> Succinyl-CoA >2> Succinate semialdehyde >3> 4HB >4> 4HB-CoA >5> poly(4HB)

#	Enzyme	Source
>1>	Succinyl-CoA:CoA transferase	*C. kluyveri* (*cat1*)
>2>	Succinate-semialdehyde dehydrogenase	C. kluyveri (*sucD*)
>3>	4HB-dehydrogenase	*C. kluyveri* (*4hbD*) *A. eutrophus* (*gbd*)
>4>	4HB-CoA transferase	*C. kluyveri* (orfZ)
>5>	PHA synthase	*A. eutrophus* (*phaC*)

(iii) Succinyl-CoA >1> Succinate semialdehyde >2> 4HB >3> 4HB-CoA >4> poly(4HB)

#	Enzyme	Source
>1>	Succinate-semialdehyde dehydrogenase	C. kluyveri (*sucD*)
>2>	4HB-dehydrogenase	*C. kluyveri* (*4hbD*) *A. eutrophus* (*gbd*)
>3>	4HB-CoA transferase	*C. kluyveri* (orfZ)
>4>	PHA synthase	*A. eutrophus* (*phaC*)

(iv) Propionyl-CoA + Oxaloacetate >1> 2-Methylcitrate >2> 2-Methylisocitrate >3> Pyruvate + Succinate >4> Succinyl-CoA >5> Succinate semialdehyde >6> 4HB >7> 4HB-CoA >8> poly(4HB)

#	Enzyme	Source
>1>	2-Methylcitrate synthase	*Saccharomyces cerevisiae*
>2>	2-Methylcitrate dehydratase plus 2-Methylisocitrate dehydratase	*Saccharomyces cerevisiae*
>3>	2-Methylisocitrate lyase	*Saccharomyces cerevisiae*
>4>	Succinyl-CoA:CoA transferase	*C. kluyveri* (*cat1*)
>5>	Succinate-semialdehyde dehydrogenase	C. kluyveri (*sucD*)
>6>	4HB-dehydrogenase	*C. kluyveri* (*4hbD*) *A. eutrophus* (*gbd*)
>7>	4HB-CoA transferase	*C. kluyveri* (orfZ)

>8> PHA synthase *A. eutrophus* (*phaC*)

8. Physiological approaches for the production of poly(4HB) homopolyesters or of copolyesters containing 4HB as one constituent from unrelated carbon sources

In addition to that said under 7. the flow of metabolites towards 4HB-CoA will be supported by applying the following strategies:

(i) If mutants lacking active 2-oxoglutarate dehydrogenase are employed 2-oxoglutarate levels in the cells will be increased and the pathway outlined under 7-(i) can be used

(ii) If mutants lacking active succinate thiokinase are employed succinyl-CoA levels in the cells will be increased and the pathway outlined under 7-(iii) can be used

(iii) If mutants lacking active succinate dehydrogenase are employed succinate levels in the cells will be increased and the pathways outlined under 7-(ii) oder 7-(iv) can be used

(iv) If enzyme of the citric acid cycle downstream from the 2-oxoglutarate dehydrogenase are specifically inhibited by chemicals added to the medium (such as e. g. malonate), the concentrations of intermediates of the citric acid cycle might be increased and one of the pathways outlined under 7 can be used.

9. "Plant specific claims"

This should be compiled by the MONSANTO specialists